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POLYMETAPHOSPHATE BASED FORMULATIONS FOR THERAPY OF MICROCRYSTALLINE ARTHROPATHIES

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The present invention relates to polymetaphosphate-based composition for therapy of microcrystalline arthropathies.

BACKGROUND ART

Microcrystalline arthropathies are a group of inflammatory-degenerative pathologies, characterized by the deposition of mineral substances in articular and periarticular structures in crystalline form. In particular, chondrocalcinosis is a disease characterized by microcrystalline deposits of calcium pyrophosphate dihydrate, $Ca_2[O(PO_3)_2](2H_2O)$ (CPPD). In the course of chondrocalcinosis, synovitic episodes secondary to the release of CPPD crystals from tissue deposits in the synovial frequently occur. The identification of crystals in the synovial liquid of patients with gout-like arthritis was described in 1962 by McCarthy [McCarthy DJ Jr, Kohn NN, Faires Js. The significance of calcium phosphate crystal in the synovial fluid of arthritis patients, the pseudogout syndrome. Clinical aspects. *Ann Intern Med* 56: 711-737 (1962)].

Another common microcrystalline arthropathy is caused by the deposit of hydroxyapatite crystals, $Ca_5(PO_4)_3OH$ (HAP), at the articular and periarticular level. Usually, this pathology manifests itself in association with other arthropathies of a pre-eminently degenerative nature such as osteoarthrosis, calcific periarthritis, tendinitis and calcific bursitis. Although calcific deposits are often not associated to specific clinical specifications, they can assume particular relevance in conditions such as calcific periarthritis of the shoulder, in which it is believed that such calcifications are partly responsible for the inflammatory degenerative manifestations of periarticular structure [Dieppe PA, Crocker P, Huskisson EC, Willoughby AD. Apatite deposition disease: a new arthropathy. *Lancet* 1: 266-268 (1976)].

The mechanism that leads to the precipitation and deposition of CPPD or HAP crystals is not yet known, nor does it appear clear whether degenerative alterations of the cartilage are primitive or secondary to the deposition of the crystals. The likeliest hypothesis is that this deposition is due to a local metabolic alteration. In case of chondrocalcinosis, the pyrophosphate produced by the chondrocytes would be diffused in the fundamental substance according to an increased synthesis or to a tissue inability to hydrolyze the compound with pyrophosphatase enzymes, including alkaline

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phosphatase. Small deposits of pyrophosphate are often observed in the cartilage of elderly subjects, especially as a result of an increased synthesis and concentration of pyrophosphates, by "nucleoside triphosphate pyrophosphohydrolase (NTPPPH) enzymes [Ryan ML, McCarthy DJ. Calcium Pyrophosphate Crystal Deposition Disease; Psedogout; Articular Chondrocalcinosis. In: *Arthritis and Allied Conditions: A Textbook of Rheumatology* (D.J. McCarthy & W.J. Koopman eds.), vol. 2 (12th Ed.), Philadelphia, Pa., Lippincott Williams & Wilkins, pp. 1835-1855 (1993)]. In turn, pyrophosphates are an important source of inorganic phosphates, which have a fundamental role in bone mineralization. There is an equilibrium between pyrophosphates and phosphates: when the former prevail, they precipitate in crystalline form; when phosphates prevail, there a greater solubilization and reduction of pyrophosphate crystals [Anderson HC. Mechanisms of pathologic calcification. *Rheum Dis Clin Am* **14**: 303-319 (1988); Rosen F, McCabe G, Quach J, Solan J, Terkeltaub R, Seegmiller JE, Lotz M. Differential effects of aging on human chondrocyte responses to transforming growth factor: increased pyrophosphate production and decreased cell proliferation. *Arthritis Rheum* **40**: 1275-1281 (1997)].

CPPD crystals have elongated rhomboidal shape, although at times they are highlighted in the shape of long or short rods and small squares, whereas HAP crystals are smaller and have needle or rod shape. Currently, it is believed that acute pseudogout attacks are due to the release into the articular cavity (synovial liquid) of CPPD crystals, which are coated (opsonized) with proteins (especially IgG) and then recognized and phagocytosed by polymorphonuclear neutrophils (PMN). During phagocytosis and the subsequent cell destruction, lysosomal enzymes, reactive oxygen species (ROS), leucotriens, are released which act as chemical mediators of the inflammation, with consequent acute arthritis or pseudogout [Burt HM, Jackson JK. Enhancement of crystal induced neutrophil responses by optonisation of calcium pyrophosphate dihydrate crystals. *Ann Rheum Dis* **52**: 599-607 (1993)]. It is supposed that shape, size and amount of the crystals play quite specific roles in PMN activation. On this subject, there are numerous studies which, while confirming the phlogogenic activity of CPPD crystals, are in poor agreement above all on the dimensions of the crystalline material able to stimulate phagocytes more intensely [Schwan et al, Schumacher HR, Fishbein P, Phelps R, Krauser R. Comparison of sodium urate and calcium pyrophosphate crystal size and other factors. *Arthritis Rheum* **18** (suppl): 783-793 (1995)].

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At the moment, only symptomatic therapies to reduce acute pseudogout attacks are available, and they are often insufficient to have a lasting effect.

The most widely used treatment for the acute form consists of performing an arthrocentesis on the inflamed articulation, possibly associated to articular washing with physiological solution and/or local infiltration of corticosteroids [Fitzgerald RH Jr. Inrasynovial injection of steroids uses and abuses. *Mayo Clin Proc* **51**: 655-659 (1976); Werlen D, Gabay C., Vischer TL. Corticosteroid therapy for the treatment of acute attacks of crystal-induced arthritis: an effective alternative to nonsteroidal anti-inflammatory drugs. *Rev Rhum Engl Ed* **63**: 248-254 (1996)].

Alternatively or in association with the aforesaid therapy, non steroidal anti-inflammatory drugs and/or colchicine, although the problem of the persistence of CPPD or HAP crystals at the tissue level still remains [Abramson SB. Treatment of gout and crystal arthropathies and use and mechanisms of action of nonsteroidal anti-inflammatory drugs. *Curr Opin Rheumatol* **4**: 295-300 (1992)].

Currently, the only prophylaxis for pseudogout attacks is the use of oral colchicine [Gonzales T, Gantes M. Prevention of acute attacks of pseudogout with oral colchicine. *J Rheumatol* **14**: 632-633 (1987); Lange U, Schumann C, Schmidt KL. Current aspects of colchicine therapy – classical indications and new therapeutic uses. *Eur J Med Res* **6**: 150-160 (2001)]. In the case of CPPD crystals, approaches have been attempted using the enzymatic route, i.e. the enzymes that are able to degrade pyrophosphates, such as yeast phosphatase and alkaline phosphatase, although these attempts have not found a valid therapeutic application, presumably due to the difficulty of preparing adequate formulations of protein origin because of antigen problems and of the high costs of production [Xu Y, Cruz T, Cheng PT, Pritzeker KP. Effects of pyrophosphatase on dissolution of calcium pyrophosphate dihydrate crystals. *J Rheumatol* **18**: 66-71 (1991); Shinozaki T, Xu Y, Cruz TF, Pritzeker KP. Calcium pyrophosphate dihydrate (CPPD) crystal dissolution by alkaline phosphatase: interaction of alkaline phosphatase on CPPD crystals. *J Rheumatol* **22**: 117-123 (1995)].

Encouraging, though not definitive, results, seem to be yielded by the oral use of magnesium carbonate, with the aim of solubilizing and inhibiting the formation of CPPD crystals [Patel KJ, Weidepsnul D, Palma C, Ryan LM, Walker SE. Milwaukee shoulder with massive bilateral cysts: effective therapy for hydrops of the shoulder. *J Rheumatol* **24**: 2479-2483 (1997)].

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In the literature, there are also anecdotal descriptions of the partial effectiveness of glycosaminoglycan polysulfate in the reduction of cartilage deposits of CPPD [Sarkozi AM, Nemeth-Csoka M, Bartosiewicz G. Effects of glycosaminoglycan polysulphate in the treatment of chondrocalcinosis. *Clin Exp Rheumatol* 6: 3-8 (1988)].

As previously mentioned, the pathogenic action of HAP crystals in the development of articular inflammatory manifestations is not quite clear, although crystalline aggregates of HAP are frequently present in articular effusions, both of inflammatory and degenerative nature, so their presence is considered an epiphenomenon. On the contrary, the action of these substances in the development of periarticular inflammatory degenerative pathologies, such as calcific periarthritis, clinically expressed in acute and/or chronic painful shoulder conditions, is well known. Currently, there are treatments aimed at the destruction and/or removal of such microcrystalline deposits such as articular washings with physiological solution and Extracorporeal Shock Wave Therapy (ESWT) [Cosentino R, De Stefano R, Selvi E, Frati E, Manca S, Frediani B, Marcolongo R. Extracorporeal Shock Wave Therapy for chronic calcific tendinitis of the shoulder: single blind study. *Ann Rheum Dis* **62**: 248-50 (2003); Ebenbichler GR, Erdogmus CB, Resch KL, Funovics MA, Kainberger F, Barisani G, Aringer M, Nicolakis P, Wiesinger GF, Baghestanian M, Preisinger E, Fialka-Moser V. Ultrasound therapy for calcific tendinitis of the shoulder. *N Engl J Med* **341**: 1237 (1999)].

In regard to the dissolution of HAP crystals, there are very few data in the literature, and they refer to the use of chemical substances that have no foreseeable therapeutic use [Doroshkin SV. Surface reactions of apatite dissolution. *J Colloid Interface Sci* **191**: 489-497 (1997)].

The lack of therapeutic treatments aimed at the dissolution of the tissue deposits of CPPD and HAP, has induced the authors to research chemical principles able to dissolve the crystals present in the articular and periarticular environment.

The activity of polymetaphosphates, antagonist to the crystallization of salts based on calcium (e.g. calcium carbonate and calcium sulfate) and other metals (e.g. iron, magnesium). This class of compounds therefore finds widespread use as softeners of hard and industrial waters, detergents in textile industries and/or dispersing agents in fabric coloring operations. In cosmetics, polymetaphosphates are particularly effective in the treatment of calcareous deposits such as tartar, they are important ingredients in anti-plaque tooth pastes [Draus F.M. et al. Pyrophosphate and hexametaphosphate

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effects in vitro calculus formation. Archs. Oral Biol. 15: 893-896 (1970); McClanahan S.F., White D.J., Cox E.R. Dentifrice compositions containing polyphosphate and monofluorophosphate. US Patent 6,190,644 (2002)].

The ability of these substances to reduce aortic calcifications in rats has been demonstrated [Fleisch H, Schibler D, Maerki J, Frossard I. Inhibition of aortic calcification by means of pyrophosphate and polyphosphate. *Nature* **207**: 1300-1301 (1965)] and skin calcification, also in rats [Schibler D, Fleisch H. Inhibition of skin calcification (calciphylaxis) by polyphosphates. *Experientia* **22**: 367-369 (1966)] and, consequently, it is possible to consider a therapeutic use aimed at solubilizing ectopic calcifications [Irving JT, Schibler D, Fleish H. Bone formation in normal and vitamin D-treated rachitic rats during the administration of polyphosphates. *Proc Soc Exp Biol Med* **123**: 332-335 (1966)].

The authors have already described the in vitro solubilizing ability of some polymetaphosphates on CPPD aggregates [Cini R, Chindamo D, Catenaccio M, Lorenzini S, Selvi E, Nerucci F, Picchi MP, Berti G, Marcolongo R. Dissolution of calcium pyrophosphate crystals by polyphosphates: an *in vitro* and *ex vivo* study. *Ann Rheum Dis* **60**: 962-967 (2001)]. However, the possible limit to the clinical use of these substances derives from the fact that:

1) the same polymetaphosphates are not uniquely identified with a definite molecular weight, since their formula is $(NaPO_3)_n$, with n which may vary from 3 to over 20;

2) crystals which are partially dissolved and reduced in volume (and possibly opsonized) as a result of an increased solubility of the pyrophosphate could be phagocytosed by PMN and macrophages with increased inflammation, additional production of ROS and start of a vicious cycle that could further aggravate the pathological condition, with persistence of phlogosis [Oyanagui Y. Role of phosphate, pyrophosphate, adenine nucleotides and sulfate in activating production of the superoxide radical by macrophages, and in formation of rat paw edema. *Agents Actions* **7**: 125:132 (1977); Swan A, Heywood B, Chapman B, Seward H, Dieppe P. Evidence for a causal relationship between the structure, size, and load of calcium pyrophosphate dihydrate crystals, and attacks of pseudogout. *Ann Rheum Dis* **54**: 825-830 (1995); Biaglow JE, Kachur AV. The generation of hydroxyl radicals in the reaction of molecular oxygen with polyphosphate complexes of ferrous ion. *Radiat Res* **148**: 181-187 (1997)].

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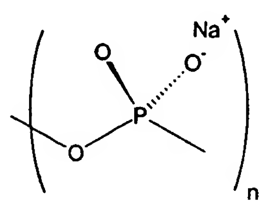
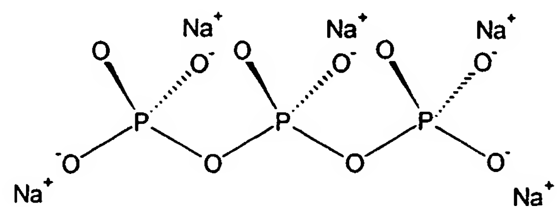
In the present invention, the above problems are solved thanks to the obtainment of formulations that contain polymetaphosphates with defined structure or salts thereof, which may be associated with one or more substances with anti-radical actions and/or with anti-oxidizing agents.

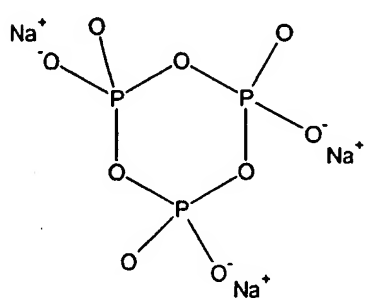
Therefore, the object of the invention is to provide a soluble pharmaceutical solution comprising an effective amount of at least one linear or cyclic polymetaphosphate or a soluble and pharmaceutically acceptable salt thereof, and appropriate diluents. Preferably, the salt of the polymetaphosphate is a sodic salt $(NaPO_3)_n$; more preferably, it is included in the following group: polymeric metaphosphate (SMP, formula a); tripolymetaphosphate (PSTP, formula b); cyclic trimetaphosphate (TSMP, formula c), cyclic hexametaphosphate (SEMP, formula d).



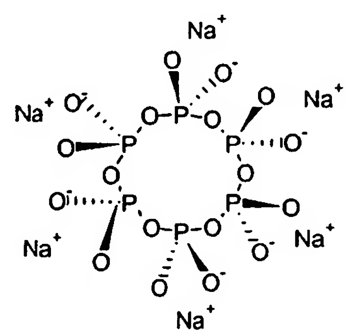
(a) SMP, $NaPO_3$ [PM = 102.0]



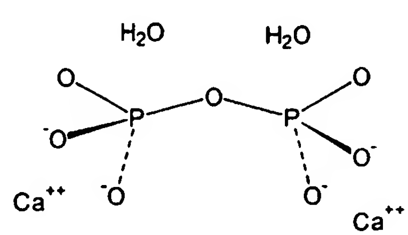
(b) PSTP, $Na_5P_3O_{10}$ [PM = 367.9]



(c) TSMP, $Na_3P_3O_9$ [PM = 305.9]



(d) SEMP, $Na_6P_6O_{18}$ [PM = 611.8]



(e) CPPD, $Ca_2P_2O_7 \cdot 2H_2O$ [PM = 290.1]

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In a preferred embodiment, the composition further comprises effective quantities of anti-oxidizers and/or ROS scavengers, such as mannitol, vitamin E, vitamin C, carotenoids, tocopherol, taurine, glucosamine sulfate, glucosamine hydrochloride. To be excluded are N-acetylcysteine, glutatione. Among them, due to their effectiveness, tolerability and simplicity of preparation are to be preferred mannitol, taurine and/or glucosamine or salts thereof are to be preferred.

Mannitol is a power scavenger of oxydryl radicals [Chaturvedi V, Wong B, Newman SL. Oxidative killing of *Cryptococcus neoformans* by human neutrophils. Evidence that fungal mannitol protects by scavenging reactive oxygen intermediates. *J Immunol* **156**: 3836-3840 (1996)]. Taurine is a power scavenger of the hypochlorite anion, of nitroxide radicals and of all ROS produced by PMN and/or activated macrophages [Park E, Alberti J, Quinn MR, Schuller-Levis G. Taurine chloramine inhibits the production of superoxide anion, IL-6 and IL-8 in activated human polymorphonuclear leukocytes. *Adv Exp Med Biol* **442**: 177-182 (1998)]. Polymetaphosphate by itself is not able to solubilize the calcium-based crystals (Ca pyrophosphates, hydroxyapatite) responsible for some arthropathies, but it is an anti-oxidizing agent that acts in synergy with known anti-oxidizers, with consequent reduction of inflammatory phenomena.

In a preferred embodiment, the formulation of the invention is also associated to one or more scavenger substances.

The obtained solutions can be injected directly into the articulations, or they can be used for continuously washing said articulations, with variable concentrations both of the polymetaphosphates and of the anti-oxidizing agents, in order to favor the solubilization of the microcrystals responsible for articulation calcification, or the reduction of inflammatory "noxa". These solutions must be isotonic, in consideration of their intra-articular use (isotony between 270 and 328 mOsmol/liter). However, it is also possible to hypothesize the use of hypo/hypertonic solutions to be used in the various therapeutic stages.

The formulation of the invention allows to inhibit the presence of ROS at the level of the articular structures produced by the phagocytosis performed by the PMN and/or macrophages at the crystalline structure level. This mechanism is responsible for oxidation stress, which is an important component of the inflammatory process, the latter being the basis for pseudogout attacks.

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The formulations, in particular those containing sodium hexametaphosphate, alone or in association with anti-radicals and/or anti-oxidizers, were tested *in vitro* to assess the ability to solubilize synthetic CPPD crystals (both monocline and tricline). The solubilization tests on the aforesaid crystals were also conducted *ex vivo* on calcified meniscii removed by arthroscopic meniscectomy from patients affected by chondrocalcinosis. Moreover, cytotoxicity tests were conducted on the solutions used on cultures of human chondrocytes.

The same formulations were tested *in vitro* to assess their solubilizing capacity on HAP crystals as well.

Each formulation, in particular those containing also anti-radicals and anti-oxidizers, was incubated *in vitro* with PMN and/or macrophages to determine with the chemiluminescence method the ability to block the production of free radicals produced by appropriately stimulated PMN. Moreover, the scavenger effect on superoxide anion, the main free radical responsible for inflammatory phenomena, was evaluated as well.

Another object of the invention is to provide a pharmaceutical formulation, injectable in intra-articular fashion, comprising a first container, containing the composition according to one of the claims 1 through 3 in powder form, and a second container, containing a solution of diluent in which is dissolved at least one substance with anti-radical action and/or one substance with anti-oxidizing action; the composition of the first container is dissolved before use. The volume of the formulation varies from 5 to 10 ml. The diluent solution can be used in association with polymetaphosphates or not, in order to exploit their anti-radical and anti-oxidizing action.

The formulation of the invention can also be used for the continuous washing of an articulation. In this case the volume of the formulation varies from 5 to 50 ml.

Within the scope of the invention is also a pharmaceutical containment formulation to be used after the solubilization of CPPD or HAP crystals in an articulation comprising a container containing a slightly hypotonic solution of dilutant, injectable in intra-articular fashion, in which is dissolved at least one substance with anti-radical and/or anti-oxidizing action. Containment formulations have a volume that may vary from 5 to 50 ml.

The invention shall now be described in its non limiting examples.

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Example 1 PREPARATION OF SOLUBILIZING SOLUTIONS IN PBS BUFFER

solutions containing polymetaphosphates, both linear and cyclic, were prepared, and pH and osmolality were measured, as shown in the following Table 1.A.

Table 1.A – Preparation of solubilizing solutions with polymetaphosphates in PBS

Solution	**Tested Polymetaphosphate**	**Preparation**	**Checked parameters**
A	Polymeric sodium metaphosphate (SMP)	500 mg of SMP were weighted and accurately added to 100 ml of PBS buffer	pH = 6.9 Isotony = 284 mOsm
B	Linear sodium tripolyphosphate (PSTP)	500 mg of PSTP were weighted and accurately added to 100 ml of PBS buffer	pH = 8.7 Isotony = 300 mOsm
C	Cyclic sodium trimetaphosphate (TSMP)	500 mg of TSMP were weighted and accurately added to 100 ml of PBS buffer	pH = 7.3 Isotony = 314 mOsm
D	Cyclic sodium hexametaphosphate (SEMP)	500 mg of SEMP were weighted and accurately added to 100 ml of PBS buffer	pH = 7.0 Isotony = 285 mOsm

Example 2 MEASUREMENT OF SOLUBILIZING ACTIVITY ON CPPD CRYSTALS

Description of the solubilization procedure and method of analysis

5 mg of synthetic CPPD crystals, both tricline and monocline (with average size 1-30 µm) were added to 5 ml of phosphate buffer without Ca^{2+} and Mg^{2+} (PBS) containing different types of polymetaphosphate at the concentration of 5 mg/ml (the four solutions mentioned in Table 1.A).

The suspension was maintained at 37°C for 1 hour under continuous agitation and subsequently filtered through 0.22 µm filters. The filtrates were subjected to analysis with spectrophotometry in atomic absorption for measurements of the final calcium concentration and the percentage of dissolution of CPPD crystals was calculated based on this data.

Solubilization results and conclusions

The results obtained can be summarized in the following Table 2.A.

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Table 2.A – Solubilizing effect on CPPD crystals after 1 hour of incubation at 37°C in PBS

Solution	Polymetaphosphate (5 mg/ml)	Dissolution (mg of CPPD/ml)	% of dissolution
a	Polymeric sodium metaphosphate (SMP)	0.344 (12)	27.5
b	Linear sodium tripolyphosphate (PSTP)	0.310 (11)	24.7
c	Cyclic sodium trimetaphosphate (TSMP)	0.023 (5)	1.9
d	Cyclic sodium hexametaphosphate (SEMP)	0.461 (12)	55.4

The results show that the solubilizing power of the examined polymetaphosphates on CPPD microcrystals can be expressed in the following order: SEMP > SMP > PSTP > TSMP.

Sodium hexametaphosphate has the greatest solubilizing activity on calcium pyrophosphate, whereas cyclic sodium trimetaphosphate has practically no solubilizing capacity.

The solubilizing capacity of sodium hexametaphosphate (SEMP) was then measured also as a function of time, observing the percentage of dissolution of CPPD at 15, 30 and 60 minutes at 37°C. The results are shown in table 2.B.

Table 2.B – Profile of the dissolving capacity of SEMP (5mg/ml) on CPPD crystals after progressively greater time intervals.

Time Minutes (37°C)	Dissolution (expressed in mg of CPPD/ml)	% of dissolution
15	0.423	50.8
30	0.451	54.0
60	0.461	55.4

The effect of sodium hexametaphosphate therefore appears to be rapid, with relevant dissolution already at 15 minutes. This results indicate a possible intra-articular use of this solution for CPPD solubilization (point number 4 of the achieved results).

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Example 3 <u>SOLUBILIZING EFFECT ON HAP CRYSTALS</u>

<u>Description of the solubilization procedure and analysis method</u>

With a method similar to the preceding example (using 8 mg of HAP crystals), the dissolving capacities of the formulations described in Table 1.A were also studied on synthetic microcrystals of HAP (10-20 μm).

<u>Solubilization results and conclusions</u>

The results obtained can be summarized in the following Table 3.A

Table 3.A – Solubilizing effect on HAP crystals after 1 hour of incubation at 37°C in PBS

Solution	Polymetaphosphate (5 mg/ml)	Dissolution (expressed in mg of HAP/ml)	% of dissolution
a	Polymeric sodium metaphosphate (SMP)	0.288 (11)	18.0
d	Cyclic sodium hexametaphosphate (SEMP)	0.150 (9)	10.0

The results show that dissolving capacity on HAP crystals is greater for SMP than for SEMP. In this case, as well, the values are relatively high and such as to program continuous washing procedures on articulations containing HAP calcifications.

The solubilizing capacity of polymeric sodium metaphosphate (SMP) was then measured as a function of time (as in the preceding example) and the results are summarized in Table 3.B.

Table 3.B. - Profile of the dissolving capacity of SMP (5mg/ml) on HAP crystals after progressively greater time intervals.

Time Minutes (37°C)	Dissolution (expressed in mg of HAP/ml)	% of dissolution
15	0.273 (11)	17.0
30	0.296 (12)	18.5
60	0.288 (11)	18.0

This result shows that a relevant dissolution is also reached after a short time (15 minutes) if compared to the maximum dissolution achieved after longer times.

Example 4 <u>CHECK OF CYTOTOXIC EFFECT ON CHONDROCYTES</u>

<u>Description of the cytotoxicity test</u>

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Samples of articular cartilage were obtained from the femoral heads of osteoarthritis patients subjected to hip prosthetization. Immediately after removal, portions of healthy cartilage were removed aseptically and 2 mm^2 fragments were washed in physiological solution with antibiotics, then digested with 1 mg/ml of clostridial collagenase in PBS with antibiotics for 14-18 hours at 37°C with moderate agitation. The solution was then filtered, washed in physiological solution and centrifuged. About 90-95% of the chondrocytes were found to be vital with the method of the Trypan blue vital dye, then pre-washed and left in plates with suitable culture medium at 37°C and 5% of CO_2.

The cells thus obtained were incubated with progressively greater concentrations of polymetaphosphates in PBS (pH 7.4) for 24 hours (6 wells for each tested concentration). The control culture was obtained incubating cells with PBS alone for 24 hours.

Cytotoxicity was determined after 1 day of exposure both with polymeric sodium metaphosphate (SMP) and with cyclic sodium hexametaphosphate (SEMP) with the tetrazole salt (MTT) method. In parallel, human chondrocytes incubated for 24 hours both with SMP and with SEMP were removed from the wells, washed in PBS; centrifuged and then fixed for 2 hours at 4°C with Kamovsky's fixative, washed in cacodilate buffer and post-fixed for one hour at 4°C with 1% of buffered osmium oxide, dehydrated and then included in resin to be subjected to sectioning with ultramicrotome. About 30 chondrocytes for each patient were examined with an electronic microscope.

<u>Results of the cytotoxic effect and conclusions</u>

The results are summarized in the following Table 4.A.

Table 4.A – Cytotoxic effect of growing concentrations of polymetaphosphates (SMP or SEMP) on human chondrocytes with the MTT method

	SMP Solutions (mg/ml)				
	0	1	2	5	15
% of metabolically active cells (mean ± SD)	100	95.0 ± 3.2	92.8 ± 4.0	63.2 ± 5.1	50.0 ± 7.6
	SEMP Solution (mg/ml)				
	0	1	2	5	15

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% of metabolically active cells (mean ± SD)	100	86.7 ± 4.6	85.2 ± 6.8	68.0 ± 5.2	48.3 ± 8.4

Values are expressed as the mean ± SD in 4 separate experiments.

The results show that the 50% inhibitory dose was reached at the highest tested concentration (15 mg/ml). In no case did morphological evaluation with the electronic microscope show cell structure alteration.

Example 5 <u>SEM AND SEMP BASED FORMULATIONS, ASSOCIATED TO COMPONENTS WITH ANTI-RADICAL AND/OR ANTI-OXIDIZING ACTIVITY</u>

<u>Pharmaceutical formulations of SEMP with anti-ROS</u>

Several pharmaceutical formulations were prepared, composed by cyclic sodium hexametaphosphate with different compounds that have ROS and hypochlorite anion scavenging capacity.

The CPPD crystal solubilizing capacity of each selected formulation was checked, to verify whether the presence of anti-oxidizing and/or anti-radical substances could inhibit the solubilization of pyrophosphate salts.

The pharmaceutical formulations are set out below:

Formulation A Components	**Concentration % (w/v)**
Cyclic sodium hexametaphosphate	1.5
Monobasic potassium phosphate	0.04
Potassium chloride	0.04
Dibasic sodium phosphate	0.23
Sodium chloride	0.65
Isotony mOsm	**297**
pH	**7.5**
Appearance	**clear**

Formulation B Components	**Concentration % (w/v)**

Cyclic sodium hexametaphosphate	0.75
Monobasic potassium phosphate	0.06
Potassium chloride	0.06
Dibasic sodium phosphate	0.345
Mannitol	3.17
Taurine	0.3
Isotony mOsm	**292**
pH	**7.5**
Appearance	**clear**

Formulation D Components	**Concentration % (w/v)**
Cyclic sodium hexametaphosphate	0.75
Monobasic potassium phosphate	0.06
Potassium chloride	0.06
Dibasic sodium phosphate	0.345
Glucosamine sulfate	2.20
Isotony mOsm	**310**
pH	**6.7**
Appearance	**clear**

Formulation O Components	**Concentration % (w/v)**
Cyclic sodium hexametaphosphate	0.5
Monobasic potassium phosphate	0.12
Potassium chloride	0.12
Dibasic sodium phosphate	0.69
Mannitol	1.55
Taurine	0.3

Isotony mOsm	**290**
pH	**7.3**
Appearance	**clear**

Formulation F Components	**Concentration % (w/v)**
Cyclic sodium hexametaphosphate	0.5
Monobasic potassium phosphate	0.06
Potassium chloride	0.06
Dibasic sodium phosphate	0.345
Mannitol	3.17
Glucosamine sulfate	0.4
Isotony mOsm	**304**
pH	**7.0**
Appearance	**clear**

Formulation L Components	**Concentration % (w/v)**
Cyclic sodium hexametaphosphate	0.5
Monobasic potassium phosphate	0.1
Potassium chloride	0.1
Dibasic sodium phosphate	0.575
Mannitol	2.64
N-acetylcysteine	0.32
Isotony mOsm	**302**
pH	**6.7**
Appearance	**clear**

Formulation N Components	**Concentration % (w/v)**

Cyclic sodium hexametaphosphate	0.5
Monobasic potassium phosphate	0.12
Potassium chloride	0.12
Dibasic sodium phosphate	0.69
Mannitol	1.55
Taurine	0.3
N-acetylcysteine	0.32
Isotony mOsm	**297**
pH	**6.6**
Appearance	**Clear**

Pharmaceutical formulations of SMP with anti-ROS

Formulation A1 Components	**Concentration % (w/v)**
Polymeric sodium metaphosphate	1.5
Monobasic potassium phosphate	0.04
Potassium chloride	0.04
Dibasic sodium phosphate	0.23
Sodium chloride	0.65
Isotony mOsm	**295**
pH	**7.4**
Appearance	**clear**

Formulation B1 Components	**Concentration % (w/v)**
Polymeric sodium metaphosphate	0.75
Monobasic potassium phosphate	0.06
Potassium chloride	0.06

Dibasic sodium phosphate	0.345
Mannitol	3.17
Taurine	0.3
Isotony mOsm	**290**
pH	**7.4**
Appearance	**clear**

Formulation D1 Components	**Concentration % (w/v)**
Polymeric sodium metaphosphate	0.75
Monobasic potassium phosphate	0.06
Potassium chloride	0.06
Dibasic sodium phosphate	0.345
Glucosamine sulfate	2.20
Isotony mOsm	**308**
pH	**6.6**
Appearance	**clear**

Formulation O1 Components	**Concentration % (w/v)**
Polymeric sodium metaphosphate	0.5
Monobasic potassium phosphate	0.12
Potassium chloride	0.12
Dibasic sodium phosphate	0.69
Mannitol	1.55
Taurine	0.3
Isotony mOsm	**287**
pH	**7.2**
Appearance	**clear**

Formulation F1 Components	**Concentration % (w/v)**
Polymeric sodium metaphosphate	0.5
Monobasic potassium phosphate	0.06
Potassium chloride	0.06
Dibasic sodium phosphate	0.345
Mannitol	3.17
Glucosamine sulfate	0.4
Isotony mOsm	**300**
pH	**6.9**
Appearance	**clear**

Formulation L1 Components	**Concentration % (w/v)**
Polymeric sodium metaphosphate	0.5
Monobasic potassium phosphate	0.1
Potassium chloride	0.1
Dibasic sodium phosphate	0.575
Mannitol	2.64
N-acetylcysteine	0.32
Isotony mOsm	**299**
pH	**6.5**
Appearance	**clear**

Formulation N1 Components	**Concentration % (w/v)**
Polymeric sodium metaphosphate	0.5
Monobasic potassium phosphate	0.12
Potassium chloride	0.12

Dibasic sodium phosphate	0.69
Mannitol	1.55
Taurine	0.3
N-acetylcysteine	0.32
Isotony mOsm	**295**
pH	**6.5**
Appearance	**clear**

Check of solubilizing capacity on CPPD crystals

The aforesaid formulations O, F, L, N containing SEMP with different compounds having anti-radical and anti-oxidizing activity were evaluated for their solubilizing capacity on CPPD crystals.

The pharmaceutical formulations O and F, containing SEMP respectively with mannitol + taurine and with mannitol + glucosamine sulfate, were found to be active in the solubilization of CPPD crystals, as shown by the results set out in the following Table 5.A.

Table 5.A – Solubilizing effect on CPPD crystals (Formulations O and F)

Incubation time (in minutes at 37°C)	**Dissolution (expressed in mg of CPPD/ml)**	**% of dissolution**
15	0.527	53.1
30	0.552	57.2
60	0.577	62.4

The pharmaceutical formulations L and N, containing SEMP respectively with mannitol + taurine + N-acetylcysteine and with mannitol + N-acetylcysteine, were found to be inactive in the solubilization of CPPD crystals, as the dissolving medium almost completely loses its potential with respect to CPPD crystals and the concentration of calcium in the filtrate is below the limit of receivability of the technique employed.

The aforesaid formulations O1, F1, L1, N1, containing SMP with different compounds having anti-radical and/or anti-oxidizing activity were evaluating for their solubilizing capacity on CPPD crystals.

The pharmaceutical formulations O1 and F1, containing SMP respectively with mannitol + taurine and with mannitol + glucosamine sulfate, were found to be active in

the solubilization of CPPD crystals, as shown by the results set out in the following Table 5.B.

Table 5.B – Solubilizing effect on CPPD crystals (Formulations O1 and F1)

Incubation time (in minutes at 37°C)	**Dissolution (expressed in mg of CPPD/ml)**	**% of dissolution**
15	0.189	20.5
30	0.214	23.2
60	0.254	27.5

The above results are surprising because they show that the selection of anti-oxidizing and anti-radical agents must be careful. For example, the presence of a power anti-oxidizer, such as N-acetylcysteine, can drastically reduce the solubilizing effect of polyphosphates.

<u>Check of solubilizing capacity on HAP crystals</u>

The aforementioned formulations O, F, L, N containing SEMP with different compounds having anti-radical and anti-oxidizing activity were evaluated for their solubilizing capacity on HA crystals.

The pharmaceutical formulations O and F, containing SEMP respectively with mannitol + taurine and with mannitol + glucosamine sulfate, were found to be active in the solubilization of HA crystals, as shown by the results set out in the following Table 5.C.

Table 5.C – Solubilizing effect on HAP crystals (Formulations O and F)

Incubation time (in minutes at 37°C)	**Dissolution (expressed in mg of CPPD/ml)**	**% of dissolution**
15	0.128	8.4
30	0.134	8.9
60	0.150	10.0

The pharmaceutical formulations L and N, containing SEMP respectively with mannitol + taurine + N-acetylcysteine and with mannitol + N-acetylcysteine, were found to be inactive in the solubilization of HAP crystals, as the dissolving medium almost completely loses its potential with respect to HAP crystals and the concentration of calcium in the filtrate is below the limit of receivability of the technique employed.

The aforesaid formulations O1, F1, L1, N1, containing SMP with different compounds having anti-radical and/or anti-oxidizing activity were evaluating for their solubilizing capacity on HA crystals.

The pharmaceutical formulations O1 and F1, containing SMP respectively with mannitol + taurine and with mannitol + glucosamine sulfate, were found to be active in the solubilization of HA crystals, as shown by the results set out in the following Table 5.D.

Table 5.D – Solubilizing effect on HAP crystals (Formulations O1 and F1)

Incubation time (in minutes at 37°C)	**Dissolution (expressed in mg of HAP/ml)**	**% of dissolution**
15	0.121	8.1
30	0.127	8.5
60	0.136	9.1

In the case of the solubilization of HA crystals, too, the selection of anti-oxidizing and anti-radical agents must be careful. For example, the presence of a power anti-oxidizer, such as N-acetylcysteine, practically eliminates the solubilizing effect of polyphosphates.

Example 6 <u>MEASUREMENT OF ANTI-RADICAL AND/OR ANTI-OXIDIZING</u>

<u>Tested pharmaceutical formulations of SEMP with anti-ROS</u>

Formulation A Components	**Concentration % (w/v)**
Cyclic sodium hexametaphosphate	1.5
Monobasic potassium phosphate	0.04
Potassium chloride	0.04
Dibasic sodium phosphate	0.23
Sodium chloride	0.65
Isotony mOsm	**297**
pH	**7.5**
Appearance	**clear**

Formulation B Components	**Concentration % (w/v)**
Cyclic sodium hexametaphosphate	0.75
Monobasic potassium phosphate	0.06
Potassium chloride	0.06
Dibasic sodium phosphate	0.345
Mannitol	3.17
Taurine	0.3
Isotony mOsm	**292**
pH	**7.5**
Appearance	**clear**

Formulation D Components	**Concentration % (w/v)**
Cyclic sodium hexametaphosphate	0.75
Monobasic potassium phosphate	0.06
Potassium chloride	0.06
Dibasic sodium phosphate	0.345
Glucosamine sulfate	2.20
Isotony mOsm	**310**
pH	**6.7**
Appearance	**clear**

Formulation E Components	**Concentration % (w/v)**
Monobasic potassium phosphate	0.08
Potassium chloride	0.08
Dibasic sodium phosphate	0.46
Glucosamine sulfate	2.20
Isotony mOsm	**312**

pH	**6.9**
Appearance	**clear**

Formulation F Components	**Concentration % (w/v)**
Cyclic sodium hexametaphosphate	0.5
Monobasic potassium phosphate	0.06
Potassium chloride	0.06
Dibasic sodium phosphate	0.345
Mannitol	3.17
Glucosamine sulfate	0.4
Isotony mOsm	**304**
pH	**7.0**
Appearance	**clear**

Formulation G Components	**Concentration % (w/v)**
Monobasic potassium phosphate	0.08
Potassium chloride	0.08
Dibasic sodium phosphate	0.46
Mannitol	3.17
Glucosamine sulfate	0.4
Isotony mOsm	**302**
pH	**7.2**
Appearance	**clear**

Formulation O Components	**Concentration % (w/v)**
Cyclic sodium hexametaphosphate	0.5

Monobasic potassium phosphate	0.12
Potassium chloride	0.12
Dibasic sodium phosphate	0.69
Mannitol	1.55
Taurine	0.3
Isotony mOsm	**290**
pH	**7.3**
Appearance	**clear**

Tested pharmaceutical formulations of SMP with anti-ROS

Formulation A1 **Components**	**Concentration %** **(w/v)**
Sodium metaphosphate	1.5
Monobasic potassium phosphate	0.04
Potassium chloride	0.04
Dibasic sodium phosphate	0.23
Sodium chloride	0.65
Isotony mOsm	**295**
pH	**7.4**
Appearance	**clear**

Formulation B1 **Components**	**Concentration %** **(w/v)**
Sodium metaphosphate	0.75
Monobasic potassium phosphate	0.06
Potassium chloride	0.06
Dibasic sodium phosphate	0.345
Mannitol	3.17
Taurine	0.3
Isotony mOsm	**290**
pH	**7.4**
Appearance	**clear**

Formulation C1 **Components**	**Concentration % (w/v)**
Monobasic potassium phosphate	0.02
Potassium chloride	0.02
Dibasic sodium phosphate	0.115
Mannitol	5.17
Taurine	0.3
Isotony mOsm	**304**
pH	**7.4**
Appearance	**clear**

Formulation D1 **Components**	**Concentration % (w/v)**
Sodium metaphosphate	0.75
Monobasic potassium phosphate	0.06
Potassium chloride	0.06
Dibasic sodium phosphate	0.345
Glucosamine sulfate	2.20
Isotony mOsm	**308**
pH	**6.6**
Appearance	**clear**

Formulation E1 **Components**	**Concentration % (w/v)**
Monobasic potassium phosphate	0.08
Potassium chloride	0.08
Dibasic sodium phosphate	0.46
Glucosamine sulfate	2.20
Isotony mOsm	**310**
pH	**6.8**
Appearance	**clear**

Formulation F1 Components	**Concentration % (w/v)**
Sodium metaphosphate	0.5
Monobasic potassium phosphate	0.06
Potassium chloride	0.06
Dibasic sodium phosphate	0.345
Mannitol	3.17
Glucosamine sulfate	0.4
Isotony mOsm	**302**
pH	**6.9**
Appearance	**clear**

Formulation G1 Components	**Concentration % (w/v)**
Monobasic potassium phosphate	0.08
Potassium chloride	0.08
Dibasic sodium phosphate	0.46
Mannitol	3.17
Glucosamine sulfate	0.4
Isotony mOsm	**300**
pH	**7.1**
Appearance	**clear**

Formulation O1 Components	**Concentration % (w/v)**
Polymeric sodium metaphosphate	0.5
Monobasic potassium phosphate	0.12
Potassium chloride	0.12
Dibasic sodium phosphate	0.69
Mannitol	1.55

Taurine	0.3
Isotony mOsm	**287**
pH	**7.2**
Appearance	**clear**

<u>Procedure for chemiluminescence produced by human PMNs</u>

Chemiluminescence [De Luca MA, McElroy WD. Bioluminescence and chemiluminescence. *Methods in Enzymol* **133**: 449-493 (1986)] is a method to evaluate the scavenger action on the pool of the ROS produced by polymorphonucleates (PMN) stimulated with zymosan [10 mg/ml of phosphate buffer without Ca^{2+} and Mg^{2+} (PBS); Sigma] opsonized according to the English method [English D, Roloff JS, Lukens JN. Regulation of human polymorphonuclear leucocyte superoxide release by cellular response to chemotactic peptides. *J Immun* **126**: 165-171 (1981)]. The PMNs were obtained from samples of peripheral venous blood of healthy subjects by centrifuging in density gradient :polymorphoprep (Nycomed), which, once centrifuged, forms a density gradient whereon the blood cells are separated.

The purity (>90%) and the vitality (>95%) of the cell population were tested by examining a strip and conducting the trypan blue exclusion test. Thereafter, to a portion (100 μl) of a suspension containing 10^6 PMN ml^{-1} of PBS, were added 100 μl of luminol (2 mg in 10 ml of NaOH 0.01M subsequently diluted 1:10 with PBS) and 10 μl of stimulator. The preparation was introduced in the chemiluminometer (Berthold Multi-biolumat LB 9505C) at 37°C; the reaction kinetics were read for 40 minutes.

All cpm values shown in the tables are extrapolated from an average of 2 values (double analysis).

For each experiment, three distinct trials were conducted.

<u>Inhibition test of the chemiluminescence produced by human PMNs relating to solutions containing SEMP in the presence or absence of other anti-oxidizing substances</u>

The results were collected in the following Table 6.A

Table 6.A – Effect on chemiluminescence of formulations containing SEMP and anti-oxidants

Formulation	**Test 1**	**Test 2**	**Test 3**
	% inhibition	**% inhibition**	**% inhibition**

Basal			
A	79.4	77.3	80.2
B	77.9	75.5	77.1
C	7.0	7.7	8.3
D	94.5	92.9	94.4
E	86.9	82.1	86.9
F	96.9	87.7	91.2
G	66.3	64.5	74.1
O	56.5	65.7	66.6

NOTE: the formulations C (taurine and mannitol), G (glucosamine and mannitol) and E (glucosamine) do not contain SEMP.

The results of the inhibition of chemiluminescence due to scalar quantities of sodium hexametaphosphate, without anti-oxidants, are instead shown in the following Table 6.B.

Table 6.B – Effect of scalar quantities of SEMP sodium (alone) on chemiluminescence

Concentration of SEMP in PBS (mg/ml)	**Test 1**	**Test 2**	**Test 3**
	% inhibition	**% inhibition**	**% inhibition**
Basal			
0.5	32.4	17.5	33.4
1	64.8	50.0	66.9
2	74.6	72.5	70.0
4	81.0	80.0	74.3
7.5	97.8	84.0	76.9

All tested formulations have shown a powerful inhibitory effect on the chemiluminescence produced by human PMNs with the procedure described above. The most amazing and unexpected was that simple solutions of sodium hexametaphosphate in PBS have shown a powerful inhibiting effect on chemiluminescence. The addition of known anti-oxidants and/or anti-radical agents allowed to maintain the inhibitory effect on chemiluminescence.

Moreover, the formulations C, E, G which do not contain SEMP must be considered the formulations for containment or rather for washing the articulation after intervening with the solutions containing sodium hexametaphosphate. These solutions must be

considered as an instrument for treating chondrocalcinosis and hence for the prophylaxis of pseudogout episodes.

Test of inhibition of the chemiluminescence produced by human PMNs relating to solutions containing SMP in the presence or absence of other anti-oxidizing substances

Table 6.C – Effect on chemiluminescence of formulations containing SMP and anti-oxidants

Formulation	Test 1	Test 2	Test 3
	% inhibition	% inhibition	% inhibition
Basal			
A1	75.9	72.5	75.0
B1	92.5	90	91.5
D1	84.9	80.1	83.9
F1	54.3	62.5	72.5
O1	77.4	75.0	78.5

The results of the inhibition of chemiluminescence due to scalar quantities of polymeric sodium hexametaphosphate, without anti-oxidants, are instead shown in the following Table 6.D.

Table 6.D – Effect of scalar quantities of SMP sodium (alone) on chemiluminescence

Concentration of SEMP in PBS (mg/ml)	Test 1	Test 2	Test 3
	% inhibition	% inhibition	% inhibition
Basal			
0.5	42.5	52.8	34
1	69.1	70	70
2	77.6	70	73.6
4	79.8	76	79.2
7.5	82	75	81.5

Formulations containing SMP have also shown a powerful inhibitory effect on the chemiluminescence produced by human PMNs with the procedure described above, with results which may be superposed with those already observed with hexametaphosphate.

Example 7 EFFECT ON THE VITALITY OF HUMAN POLYMORPHONUCLEATES (PMN)

Method for determining PMN vitality

The solutions were prepared solubilizing the sodium hexametaphosphate in PBS and adding PMNs ($1x10^5$/ml), obtained from venous blood of healthy volunteers. Incubation was performed at 37°C for 5 minutes. Subsequently, Trypan was added and the cells were observed with the microscope, calculating the number of vital cells.

Tests with SEMP

The vitality of the PMNs in contact with solutions containing scalar quantities of sodium hexametaphosphate was tested, in the presence or absence of the same anti-oxidants and/or anti-radical agents for chemiluminescence inhibition tests. For each concentration, pH and osmolality were measured as well (the pH of all solutions was brought back to 7.5). The results are shown in Table 7.A.

Table 7.A

Concentration of SEMP in PBS (mg/ml)	**pH**	**Osmolality (mOsm)**	**% Vitality PMN**
0.5	7.5	273	100
1	7.5	274	97
2	7.5	274	96
4	7.5	280	92
7.5	7.5	294	80
15.0	7.5	322	75

None of the tested concentrations caused a marked reduction in PMN vitality, except for the maximum tested concentration (15 mg/ml).

The experiment was repeated using formulations containing hexametaphosphate and various anti-oxidants (see Example 6), without harmful effects on PMN survival. The results are shown in Table 7.B.

Table 7.B

Formulation	**pH**	**Osmolality (mOsm)**	**% Vitality PMN**
A	7.5	297	98
B	7.5	292	99

C	7.5	306	98
D	6.7	310	98
E	6.9	312	97
F	7.0	304	93
G	7.2	302	98
L	6.6	302	91
N	6.6	297	97
O	7.3	290	97

Tests with SMP

The vitality of the PMNs in contact with solutions containing scalar quantities of sodium metaphosphate was tested, in the presence or absence of the same anti-oxidants and/or anti-radical agents for chemiluminescence inhibition tests. For each concentration, pH and osmolality were measured as well (the pH of all solutions was brought back to 7.5). The results are shown in Table 7.C.

Table 7.C

Concentration of SEMP in PBS (mg/ml)	**pH**	**Osmolality (mOsm)**	**% Vitality PMN**
0.5	**7.5**	**268**	**99**
1	**7.5**	**269**	**97**
2	**7.5**	**271**	**98**
4	**7.5**	**282**	**93**
7.5	**7.5**	**292**	**84**
15.0	**7.5**	**320**	**74**

None of the tested concentrations caused a marked reduction in PMN vitality, except for the maximum tested concentration (15 mg/ml).

The experiment was repeated using formulations containing metaphosphate and anti-oxidants (see Example 6), without harmful effects on PMN survival. The results are shown in Table 7.B.

Table 7.B

Formulation	**pH**	**Osmolality (mOsm)**	**% Vitality PMN**

A1	7.4	295	96
B1	7.4	290	97
C1	7.4	304	99
D1	6.6	308	95
E1	6.8	310	98
F1	6.9	302	90
G1	7.1	300	98
L1	6.5	299	88
N1	6.5	295	96
O1	7.2	287	94

Example 8 MEASUREMENT OF SUPEROXIDE ANION INHIBITION

Method for determining superoxide anion

The production of O_2 by stimulated PMNs [in this case, stimulation was conducted with Phorbol 12-myristate 13-acetate (PMA)], was evaluated through the reduction of the cytochrome-C, as described in English's method [English D, Roloff JS, Lukens JN. Regulation of human polymorphonuclear leucocyte superoxide release by cellular response to chemotattic peptides. *J Immun* **126**: 165-171 (1981)]. For this purpose, to a portion of 750 μl of PBS were added, in this order: 100 μl of cytochrome-C (30 mg/ml), 100 μl of stimulator and 100 μl of cellular suspension. The preparation was incubated for 25 minutes at 37°C; subsequently, 50 μl of superoxide dismutase (SOD) 1 mg/ml, 75000 units (Sigma) to stop the reaction, lastly centrifuging for 10 minutes at 4°C and a spectrophotometric reading (Beckman DU6) of the surnatant at 550 and 468 nm. The "white" was prepared introducing the SOD in a sample before all other reactants. The PMNs were prepared as described previously, the stimulator (PMA) was prepared as described in English's method. The results are expressed in nMoles/10^6 PMNs.

It is interesting to note that the scavenger effect on superoxide anion is directly proportional to the concentration of only hexametaphosphate in PBS and it is readily apparent at the concentration of 5 mg/ml. The addition of anti-oxidants like mannitol and taurine (Formulation O with 0.5mg/ml SEMP) considerably modified the anti-oxidizing activity of hexametaphosphate, alone at equal concentration.

Tests with SEMP

The results are summarized in Table 8.A

Table 8.A

Table 8.A Formulations	Test 1	Test 2
	% inhibition	% inhibition
Basal		
PBS + SEMP 0.5 mg/ml	**12.5**	**14.0**
PBS + SEMP 1 mg/ml	**30.8**	**38.8**
PBS + SEMP 2 mg/ml	**43.7**	**47.4**
PBS + SEMP 5 mg/ml	**53.1**	**56.2**
Formulation O	**78.6**	**74.7**
Formulation E	**75**	**70**
Formulation G	**69.7**	**79.7**

Unexpectedly, hexametaphosphate showed an inhibitory power on the production of superoxide anion, in direct proportion to its concentration. The presence of other anti-oxidizing or anti-radical substances enhances said inhibiting effect.

The experiment of the superoxide anion show, more than was already demonstrated by the chemiluminescence experiment, the extreme importance from the therapeutic viewpoint and the high degree of innovation from the patent viewpoint, of the association of polymetaphosphates with anti-oxidizing and/or anti-radical substances.

Moreover, the formulations C, E and G can also be considered the formulations for the containment or rather the washing of the articulation after intervening with solutions containing sodium hexametaphosphate. It can be considered as a point reached for containment solutions.

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